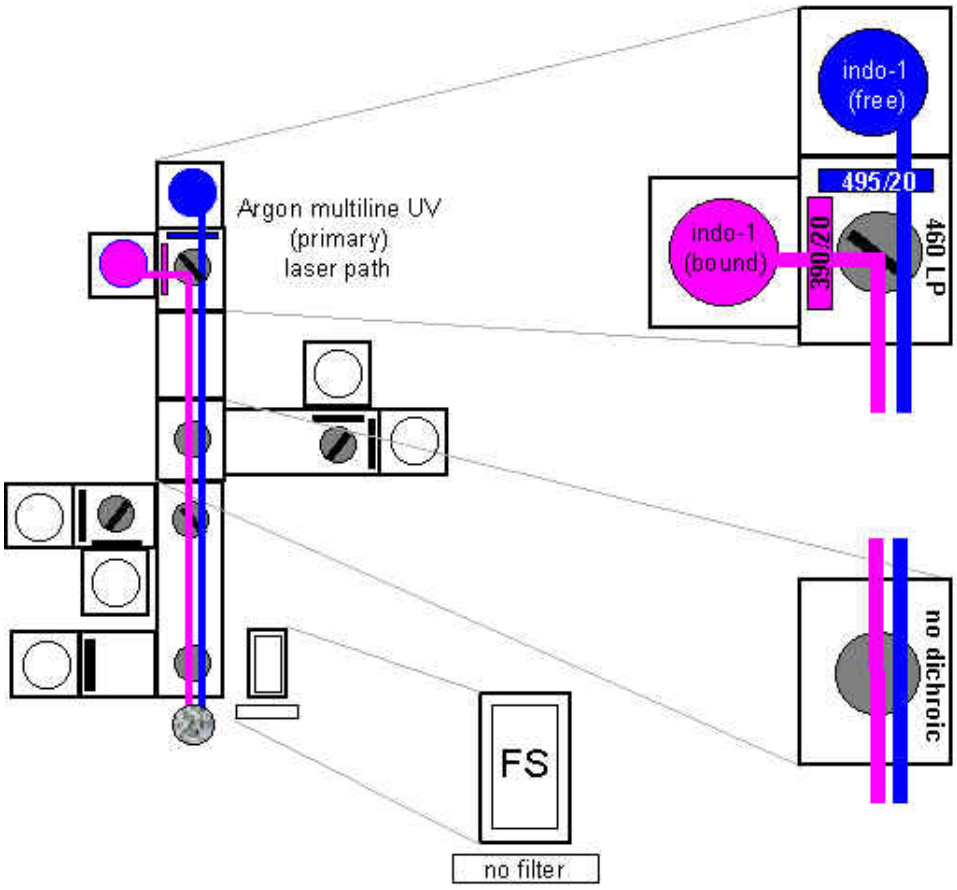
NCI ETI Branch
Flow Cytometry Core Laboratory

Measurement of calcium flux on the FACSVantage SE using indo-1.

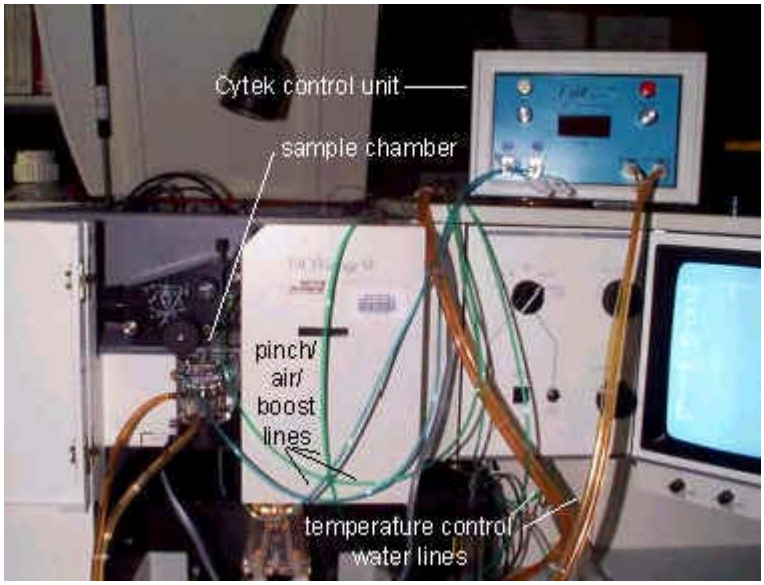
The ratiometric calcium probe indo-1 (AM ester form) can be loaded into cells for analysis of calcium flux following cell stimulation. Detection of the ratio between bound indo-1 and free indo-1 (and hence the relative intracellular calcium concentration) can be carried out on the FACSVantage using a UV laser source for indo-1 excitation and 390/20 and 495/20 nm narrow bandpass filters for detecting bound and free indo-1 fluorescence respectively. We can detect indo-1 using **one of two bench configurations.**

In the first, we use our **argon-ion laser in the primary position tuned to muliline UV output** (100 mW) as an excitation source. The free and bound forms of indo-1 are then detected in the FL1 and FL2 PMTs (normally FITC and PE) using the above bandpass filters and a 460 longpass dichroic with extended transmission to split the signals. All other dichroics in the signal path are removed. Although a 380 longpass dichroic can be used to split a side scatter signal (as we do with AMCA detection off the primary laser), we have found that it diminishes the 390 nm signal strength somewhat; hence, we omit it. The 488/10 bandpass filter is removed from the forward scatter photodiode to facilitate scatter detection using the UV laser. Alignment is carried out using Hoechst 33342-labeled chicken erythrocytes or Polyscience Blue Beads. We use a Cytek Time Zero kinetic module to add reagents to cells on-line.

(Below). Optical bench configuration for detection of indo-1 *using the primary beam path*. Indo-1 is excited with the primary argon-ion laser tuned to multiline UV. 390/20 and 495/20 nm narrow bandpass filters (custom-fabricated by Chroma Technology) are used to detect bound and free indo-1 respectively, the two signals split by a 460 longpass dichroic (fabricated with extended transmission to the 390 nm range). All other dichroics are removed from the optical path and the openings capped. The 380 LP dichroic normally used for SS detection off the UV laser is omitted for indo-1 as it reduces sensitivity. The 488 nm filter is also removed from the forward scatter photodiode.

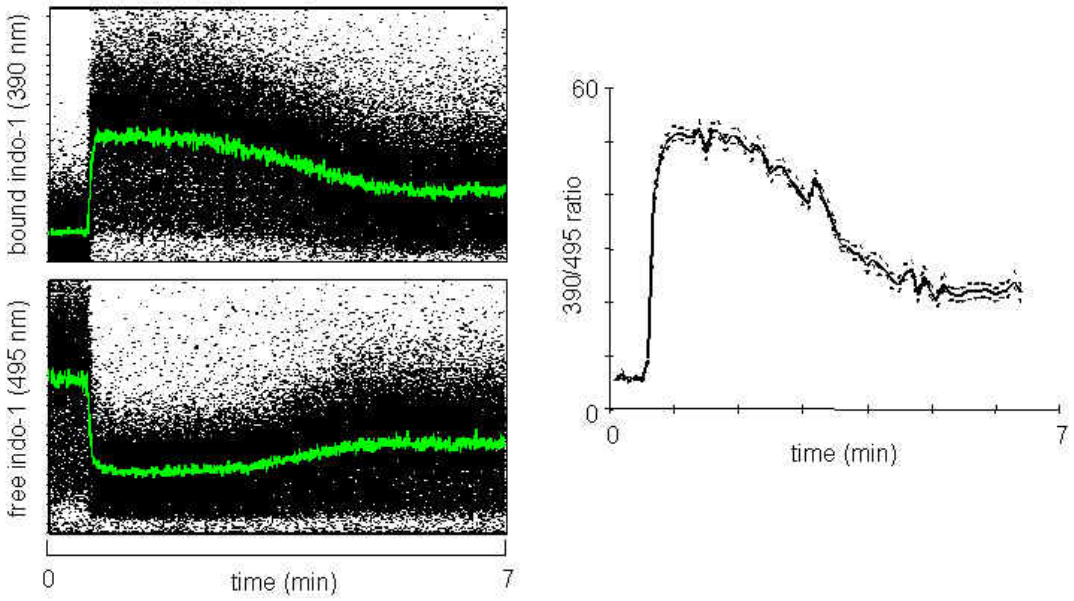


(Below). Our Cytek Time Zero kinetic module set up on our FACSVantage SE. We use the instruments air supply to provide sample chamber normal and boost air.



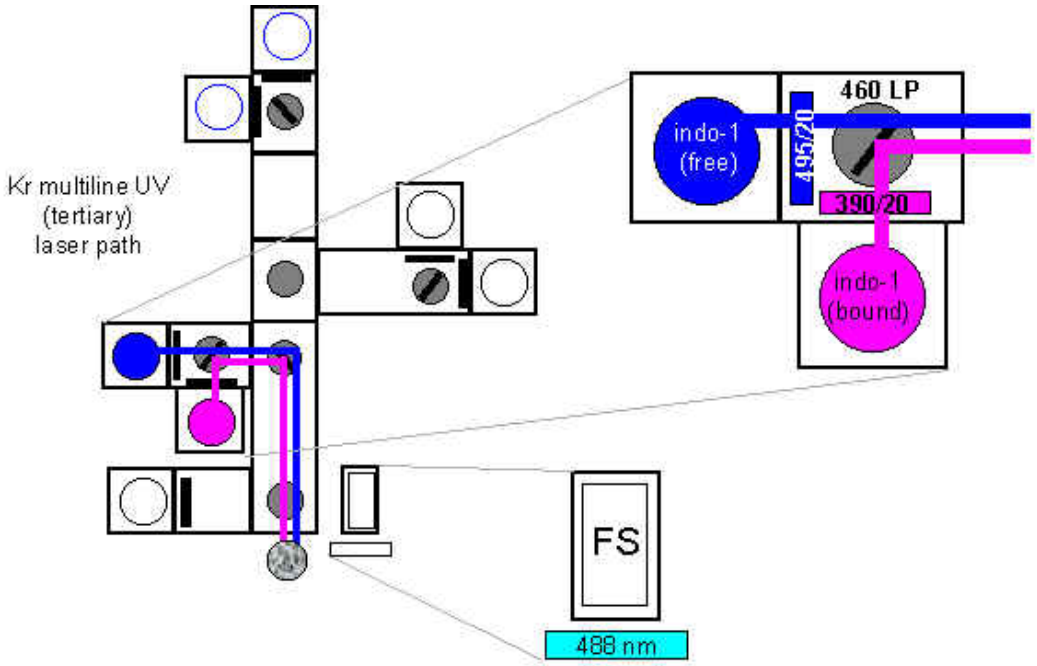
(Below). Human monocytes loaded with indo-1 AM (Molecular Probes) at 3 uM at 37 degrees centigrade for 30 minutes and analyzed on the FACSVantage using the above filter configuration.

Ionomycin at 1 µM was added to the cell population on-line using the Cytek Time Zero kinetics module at 30 seconds following the start of data acquisition. The dotplots on the left show the fluorescence emission at 390 nm (bound indo-1) and 495 nm (free indo-1). Both wavelengths were collected in linear scale. The plot on the right shows the ratio between 390 and 495 nm.

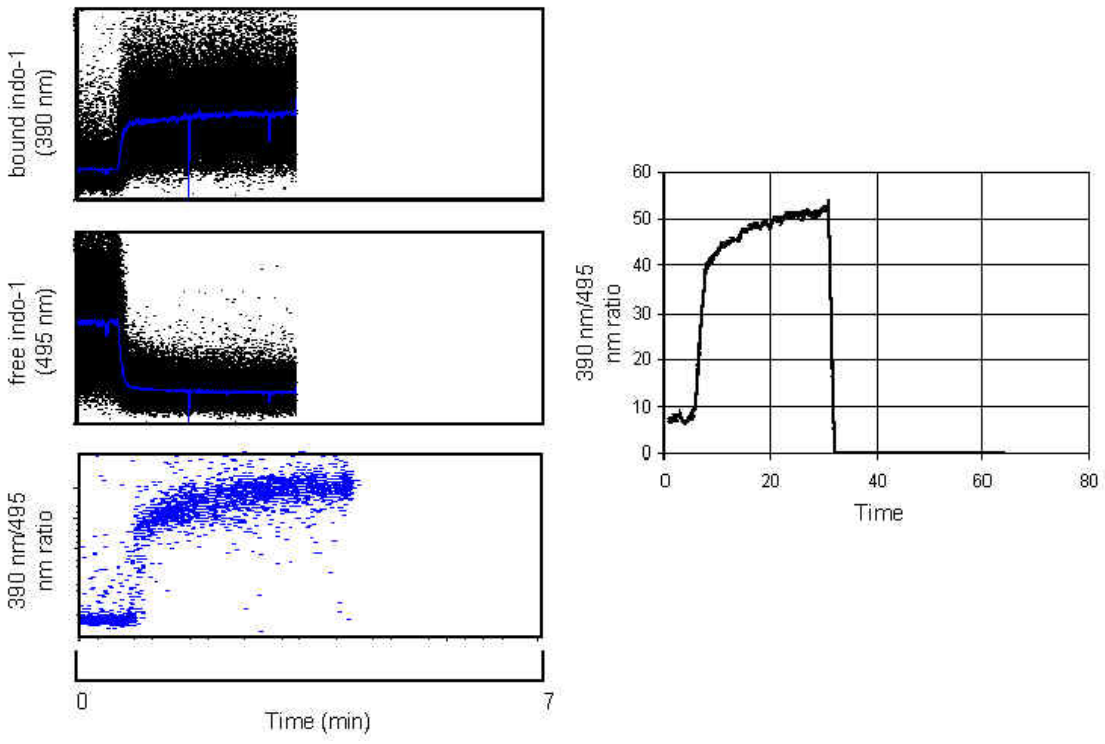


With the installation of our FACSVantage SE upgrade (with three beam paths), **we can now excite indo-1 off the tertiary krypton-ion laser set to UV multiline**, retaining the primary beam at 488 nm for forward scatter and simultaneous immunophenotyping. The indo-1 filters are the same as those used for primary laser indo-1 detection. The distance between primary and tertiary signal paths permits usae of the primary laser at 488 nm with little laser light spillover into the teritiary PMTs.

(Below). Optical bench configuration for detection of indo-1 *using the tertiary beam path*. In this setup, indo-1 is excited with the tertiary krypton-ion laser tuned to multiline UV. The 390/20 and 495/20 nm narrow bandpass filters described above are used to detect bound and free indo-1 respectively, the two signals split by the 460 longpass dichroic. Both signals are diverted to the tertiary PMTs with a 3-way 100% mirror. Special attention needs to be paid to proper laser alignment, since primary 488 nm laser light can spill over into the tertiary path PMTs. This spillover should not occur if the laser beams and signal paths are sufficiently separated. Side scatter can be detected using the primary 488 nm beam, although the partial reflectance mirror usually used to divert the side scatter signal can be removed to improve indo-1 detection sensitivity. . Forward scatter can be detected as normal with the primary laser and a 488/10 nm filter.

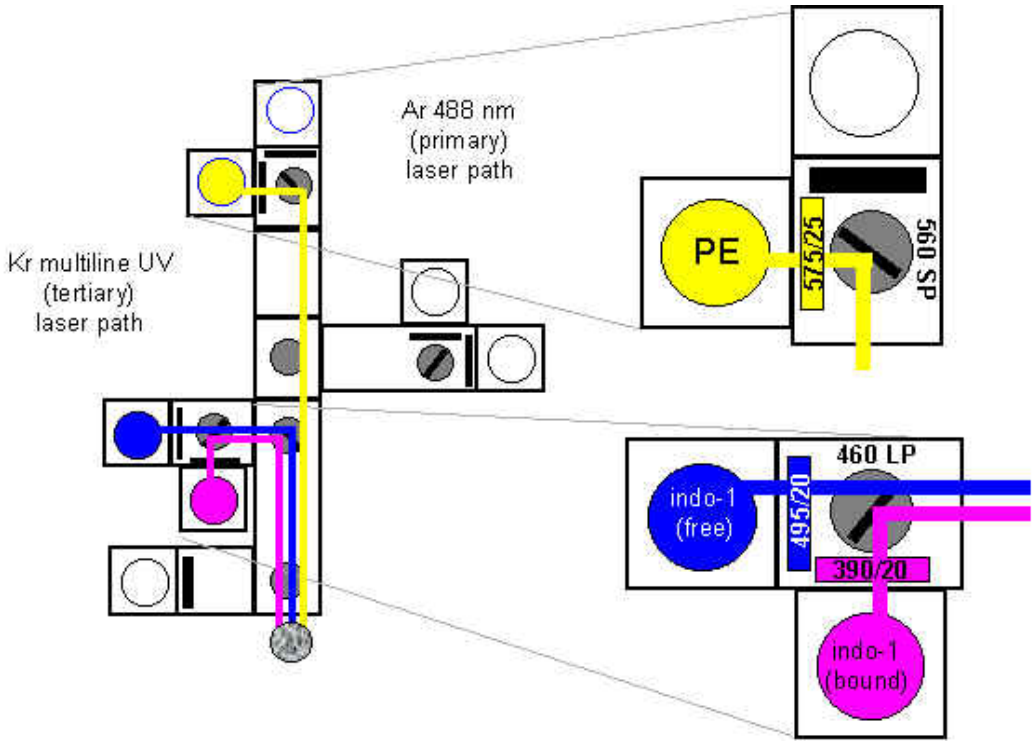


(Below). Murine EL4 cells were loaded with indo-1 AM at 5 uM at 37 degrees centigrade for 30 minutes and analyzed on the FACSVantage using the above filter configuration. Ionomycin at 1 µM was added to the cell population on-line using the Cytek Time Zero kinetics module at 30 seconds following the start of data acquisition. The dotplots on the left show the fluorescence emission at 390 nm (bound indo-1), 495 nm (free indo-1) and the ratio between the two wavelengths. Both wavelengths were collected in linear scale. The plot on the right shows the ratio between 390 and 490 nm.

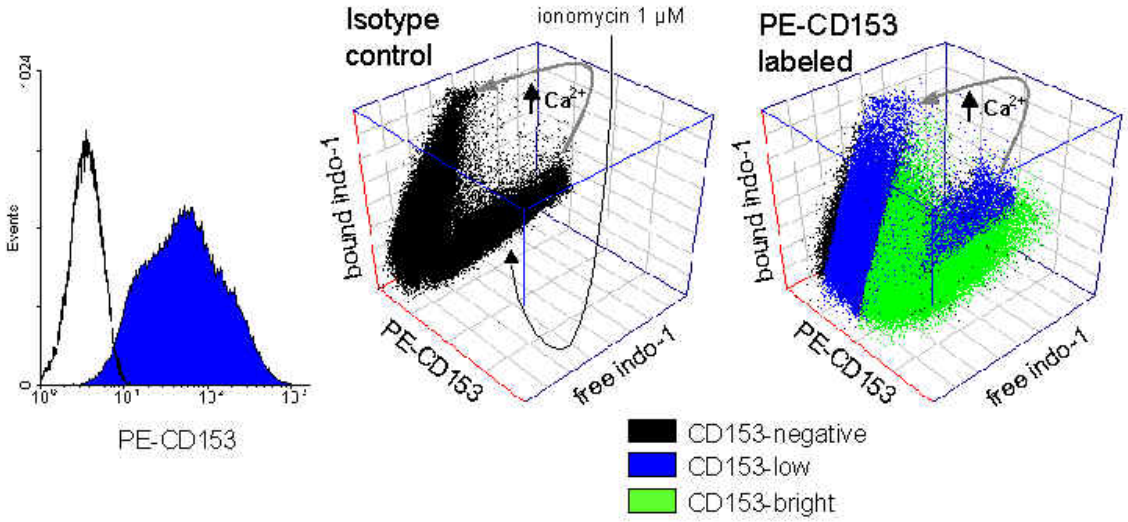


Excitation of indo-1 off the krypton tertiary laser allows us to use the primary laser to excite one or more PMTs. FITC is difficult to use with indo-1 since their emission spectra overlap, resulting in signal crosstalk; however, PE, PE-Cy5 and APC can all be used as immunolabeling fluorochromes simultaneously with indo-1 (APC off the HeNe 632 nm beam). Phenotype-specific calcium flux can then be measured.

(Below). Optical bench configuration for detection of indo-1 using the tertiary beam path and PE using the primary laser. This setup if identical to the one above with the addition of PE analysis in FL2.



(Below). Murine EL4.153.1 (CD153 sort-selected) cells were loaded with indo-1 at 5 uM at 37 degrees centigrade for 30 minutes, washed and subsequently immunlabeled with PE-anti-CD153 (Caltag) for 20 minutes. The cells were then analyzed on the FACSVantage using the above filter configuration. The histogram on the left shows the PE-CD153 fluorescence, being positive for the entire population but varying from low to high. The 3-D dotplots (middle, isotype control; right, PE-CD153) show PE-CD153 expression on the X-axis, and bound and free indo-1signals on the Z- and Y-axes respectively). Addition of ionomycin results in a signal shift from free to bound indo-1; calcium flux in CD153 low (blue) and high (green) subpopulations is clearly visible.



Click here to see indo-1 done on the B-D LSR, a benchtop flow cytometer equipped with a HeCad UV laser.